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PRINCIPAL INVESTIGATOR: Gabriela S. Dveksler, Ph.D.

CONTRACTING ORGANIZATION: Henry M. Jackson Foundation for the Advancement of Military Medicine
Rockville, MD 20852

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Gabriela S. Dveksler, Ph.D.

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Pregnancy specific glycoproteins (PSGs) are secreted proteins of unknown function that belong to the carcinoembryonic antigen gene family (CEA). CEA is a commonly used tumor marker for adenocarcinomas. Expression of PSGs in normal breast tissue is undetectable by current techniques but they have been shown to be expressed in certain tumors including breast ductal and lobular carcinomas by immunohytochemistry. Because polyclonal anti-PSG antibodies can cross react with other members of the CEA family, we have examined the expression of PSGs by reverse transcriptase-polymerase chain reaction with PSG-specific primers. Our results indicate that several breast cancer cell lines expressed mRNA encoding for PSGs and their splice variants. When breast tumors were examined, 24 out of 81 tumors expressed PSGs. We are currently looking at which particular PSGs are expressed in these tumors and will attempt to extract RNA from formalin-fixed breast tumors to extend our studies. We have produced one recombinant PSG containing the integrin binding motif, RGD, and one lacking the RGD tripeptide in insect cells. These proteins were produced as fusions with glutathione-S-transferase and have the predicted molecular mass. These proteins will be used to determine the possible effects of PSGs in the rate of proliferation of breast derived cells.

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Gabriella Drebler 8/20/97

PI - Signature Date

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INTRODUCTION

Pregnancy specific glycoproteins (PSGs) are a subfamily of secreted proteins within the carcinoembryonic family of unknown function. These proteins are derived from 11 closely related genes organized in tandem in chromosome 19. In addition to the 11 different mRNAs that are encoded by the 11 PSG genes, the number of PSG mRNAs is increased due to the presence of alternative splicing and the use of alternative polyadenylation sites. They have been classified as type I (three immunoglobulin-like constant domains) and type II (two Ig-like constant domains). All PSG splice variants conserve the leader peptide followed by the N-terminal domain. This domain has the structure of the immunoglobulin variable-like domains. Some PSGs such as PSG 6 and PSG 11 have the tripeptide sequence Arg-Gly-Asp (RGD) in the N-domain while others like PSG 1 do not. The RGD sequence is present in a variety of extracellular matrix proteins that bind to integrin receptors such as fibronectin and vitronectin. The extracellular matrix regulates many aspects of cell phenotype including differentiation state, control of gene expression, apoptosis, and cell proliferation. In mammary epithelial cells, the basement membrane has a central role in controlling gene expression and this process is mediated by a signal transduction cascade involving integrin receptors.

Normal breast tissue does not express PSGs as indicated by the lack of amplification of PSG cDNAs by reverse transcriptase (RT) polymerase chain reaction (PCR). Elevated levels of PSGs have been reported for some tumors which include choriocarcinomas, chorioepitelioma, invasive and hydatidiform mole, and breast carcinomas. Published reports vary substantially in the accounted percentage of breast tumors that present with elevated PSG levels. The discrepancies can be accounted for by different factors: the anti-PSG antibodies used for the study, the fixation technique employed, the sensitivity of the technique, and the difference in secretory capacity between tumors when serum levels were measured.

We proposed to :
1. Analyze pregnancy specific glycoprotein mRNA expression in human breast cancer cell lines and breast cancer tissue.
2. Transfect the selected PSG cDNAs in breast cancer cell lines and establish stably transfected cell lines.
3. Obtain large quantities of purified PSG for future functional studies.

PROGRESS REPORT
**Results obtained from 8/96 to 7/97**

Analysis of pregnancy specific mRNA expression in human breast cancer cell lines:
We have obtained human cell lines derived from breast tissue from the American Type Culture Collection. These include cell lines that are estrogen receptor positive: MCF-7, T47D, ZR-75-1, and cell lines that are estrogen receptor negative: Hs578 T, BT 474, BT 483. We also obtained the cell line Hs578 Bst that is derived from normal marginal tissue and the cell line MCF10a which is derived from a spontaneously immortalized breast

epithelial line. Total RNA was prepared from the cell lines listed above using the Trizol reagent (Life Technologies) at least twice. To analyze the expression of the different PSGs and its splice variants we amplified the cDNAs with three primers sets specific for human PSGs. These primer sets have been previously published and do not cross react with other members of the carcinoembryonic antigen family (1), (2), (3). The table below shows the primer sequences, the position of the primers, and the size of the expected PCR products. In the case of the third primer set, the two sizes reflect the amplification of the full length PSG and its splice variant lacking one constant Ig-like domain. The sequence of the $^{32}P$ end labeled oligonucleotide used as a probe for the Southern blots of the PCR reactions performed with each primer set is also included. Note that some of the primers were used as probes when the oligonucleotide sequence was between that of the primers. To monitor the quality of the RNA preparations prior to amplification of the PSGs, we amplified the house keeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or hypoxantine ribosyl transferase (HPRT) using 500 ng of RNA for 25 cycles. The RNA preparation was considered satisfactory when the 260/280 ratio was between 1.6 to 2 . In all cases, 10% of the PCR reactions were separated on a gel next to a molecular weight standard and the products were visible after ethidium bromide staining.

| Primer sequence | Position | Probe sequence | Size of PCR product |
|---|---|---|---|
| 5'AGTTTCTGGATCCTAGGCT (s1) | exon 1(leader) | | 298 bp |
| 5'GATGTAATGTAATGGTAGAG (as1) | exon 2 (L/N) | CCCTC(CT)CAGCCCCT (CT)CCTGCAC | |
| 5'CCCTC(CT)CAGCCCCT(CT)CCTGCA C(s2) | exon 1(leader) | | 300bp |
| 5'GATCAGCAGGGATGC(AG)TTGGAA( as2) | exon 2 (L/N) | TTCTACTTGTCCACA ATTTGCC | |
| 5'CTTCTACTTGTCCACAATTTGCC(s3) | exon 2 (L/N) | | 854 or 575 bp |
| 5'CTGGGGAGGTCTGGACCAT (as3) | exon 5 (B2) | CCCTGCTGATCCAGAA TGTCAC | |

L/N indicates the leader/N-domain exon, (s) indicates sense primer as (as) indicates antisense primer. The size of the expected product is indicated next to the sequence of the sense primer of the set while the sequence of the probe is indicated next to the primer set's antisense primer. Each set is identified by a number. For example, (s1) and (as1) indicate one of the primer sets used for the amplifications while (s2) and (as2) represent a different primer set.

To determine which specific PSGs were expressed, we amplified the cDNAs with the universal primer that anneals to the B2 domain (exon 5) of all PSGs listed in the table above in combination with the specific PSG sense primers listed in the table below as previously published by Wu and co-workers (1). This primer combination allows for the identification of type I versus type II splice variants. To increase the sensitivity of the assay, after the amplification 10-20 ul of the PCR reaction were separated on a gel and

transferred to a Nytran membrane. Membranes were then hybridized to a probe that hybridizes to all PSGs in the second exon which corresponds to the N-terminal domain of the glycoproteins.

| Primer sequence | PSG that is amplified |
|---|---|
| ATCATATGTAGTAGACGGTGAAATA | PSG 1 |
| GCCATGGAAACTGTGATCTTAAC | PSG 2 |
| ATCATACGTAGTAGATGGTCAAAATAA | PSG 3 |
| ACAAAGGGCAAATGACATACGTC | PSG 4 |
| TACAAAGGACAACTGATGGACC | PSG 5 |
| CAAATTATATATGGGCCTGCCTA | PSG 6 |
| CATTATATCGTATATAGTTGATGGTAA | PSG 11 |

Different amounts of RNA from the breast cancer cell lines were tested for the reverse transcriptase reaction, going from 1 up to 3 ug. To perform the cDNA reactions we used the Ready to Go U-prime beads from Pharmacia Biotech with the appropriate antisense primer. Amplification of the cDNAs was performed with the three primer sets indicated above for different number of cycles (30 and 35). Controls for contamination with no template added were always included. Reactions were also performed to identify the specific PSGs expressed in the cells. These reactions were carried out in two ways. The cDNAs were first amplified with a universal primer set, and were then reamplified for 21 cycles with a primer set specific for each PSG (1) or the cDNAs were amplified with the PSG- specific primer set for 35 cycles with no reamplification.

We found that all the cell lines tested expressed PSGs. Interestingly, besides the expected size fragments a smaller size product was detected using the exon 2-exon B2 primer set. This product, that hybridized to the oligonucleotide probe, could represent the type III splice variant which lacks the A1 and A2 constant Ig-like domains first described by Teglund and co-workers for PSG 11 (2). The cell line MCF10a was reported not to express mRNA encoding for PSGs (1). Our result may differ from the published one because we have not only examined the presence of the amplification product by ethidium bromide staining but we have also detected the product with a $^{32}P$ labeled probe. Hybridization with a radioactive probe increases the sensitivity of the detection considerably. Furthermore, in same instances, the signals were detected after 3 days of exposure at -70 degrees. Therefore, the expression levels in same of the cell lines are likely to be very low.

Analysis of pregnancy specific mRNA expression in human breast cancer tissue:
Our original plan was to obtain autopsy material for RNA preparation from the NNMC. This has proven difficult because the tissue needs to be snap frozen in liquid nitrogen almost immediately to assure the extraction of good quality RNA. To circumvent this problem, we will prepare RNA from formalin-fixed paraffin-embedded human breast tissue with the method described by O'Driscoll and co-workers (4). We have obtained

several lobular and ductal adenocarcinoma in paraffin from NNMC that we will start to analyze for PSG expression with the published protocol.

To overcome the problem of availability of tissue during the first year, we set up a collaboration with Dr. J. Thompson from the Univ. of Freiburg, Germany. His laboratory has sent us total RNA from 22 invasive lobular carcinomas and 2 invasive ductal carcinomas for our analysis. These samples that were selected from 81 breast tumors were shown to express CEA-related family members by PCR in his laboratory. We amplified 1 ug of RNA from these tumors for 35 cycles and demonstrated that one of the CEA family members expressed in all these tumors was PSG. Unfortunately, we have very small quantities of RNA left but we will attempt to amplify the tumor tissues to determine the expression of particular PSGs and their splice variants.

Transfect the selected PSG cDNAs in breast cancer cell lines and establish stably transfected cell lines.

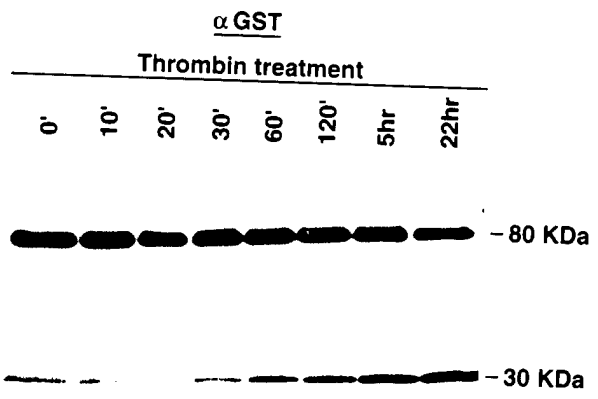
Because we could not find a breast cancer derived cell line that does not encode for PSGs so far, we will modify our original proposal. Although the mRNA for PSGs was detected, the levels of expression are low as determined by the number of cycles needed to detect the message and the need to perform a Southern blot for the detection of the product in same cases. Is important to realize, however, that we have no information regarding the translation of the mRNA and whether or not the proteins are produced and secreted.

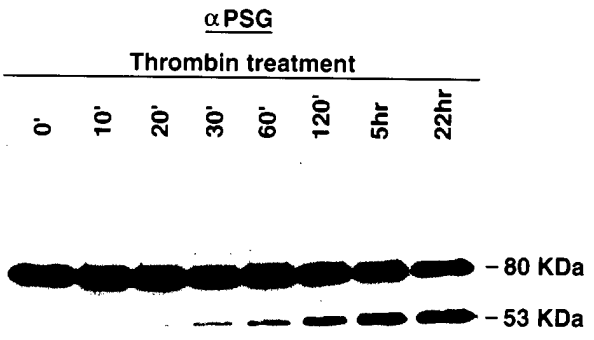
To determine the possible effect of PSGs in the growth of these cells, we generated recombinant PSGs in a baculovirus expression system as originally proposed as our third task. Rather than transfecting cells that do not normally express PSGs with the cDNAs, we will treat the cells with the recombinant proteins and measure the possible effect of these proteins on the cell's growth rate. PSG genes have been divided into class one and class two. This division is based on the differences observed in the promoter sequences (5). Class one genes include; PSG 1, PSG 3, and the pseudogene PSG 12 while class 2 genes include PSG 5, PSG 6, and PSG 11. Another classification of PSGs is based on the presence of the RGD tripeptide sequence in the N-terminal domain. Some members of the family such as PSG 11 contain the RGD sequence while others like PSG 1 do not. We therefore decided to express one PSG of each class and one protein with (PSG11) and one without the RGD sequence (PSG1). The selection of the PSGs to express as recombinant proteins in insect cells was also based on preliminary results we have obtained from two breast tumors obtained from Dr. Thompson in which these 2 PSGs were expressed. The next section will describe in detail how these proteins were generated and purified to homogeneity.

Obtain large quantities of purified PSG for future functional studies:

Because PSGs are proteins that are secreted from the cell, we wanted to be assured that these human proteins were going to be processed and secreted from the insect cells into the serum free medium. Therefore, we replaced the PSG leader peptide which may not be efficiently recognized by the insect cell machinery for that of the leader peptide of a baculovirus protein, gp67, that is normally secreted from the insect cells. We also wanted

to have a way to purify the proteins to homogeneity without having to go through a denaturation step. For this purpose, the PSG cDNAs were cloned into the baculovirus expression vector pAcSecG2T that is commercially available from Pharmingen. This vector contains both the gp 67 leader peptide followed by the glutathione-S-transferase gene (GST) gene. To clone the PSG cDNAs in frame into the Bam HI-EcoRI sites of the multiple cloning site of this vector, we designed a set of PCR primers containing the Bgl II and Eco RI restriction enzyme recognition sequences in the 5'or sense primer and the 3'or antisense primer respectively. The 5' primers started at the triplet corresponding to the first amino acid of the mature proteins while the 3' primers ended at the amino acid encoding the stop codon. Recombinant baculovirus expressing PSGs were obtained by several rounds of plaque purification. To determine the best conditions for protein production, Hi 5 insect cells were infected with the recombinant baculoviruses at a multiplicity of infection of one, and supernatants were harvested at different time point post infection. The supernatants were then analyzed on an SDS-PAGE gel and were immunoblotted with the anti-GST antibody. We performed subsequent infections to upscale protein production and harvested the supernatants at 72 hours which was the time point that gave us the largest amount of protein with minimal or no protein degradation products. To obtain PSG 1 and 11 purified to homogeneity, supernatants of infected cells were harvested, purified with glutathione-agarose beads (Sigma) and eluted with 5mM reduced glutathione. The proteins were then run on an SDS-PAGE and immunoblotted with an anti-GST antibody (Pharmingen), a polyclonal anti-PSG antibody (DAKO) and a monoclonal anti-PSG antibody (BAP 1) obtained from Dr. Zimmerman. All three antibodies detected proteins of the calculated molecular mass corresponding to PSG 1 and PSG 11. To verify the presence of carbohydrates in these proteins, we then immunoprecipitated the supernatant of insect cells infected with the recombinant viruses with concavalin-A-agarose beads followed by immunoblotting with the monoclonal anti-GST and BAP 1 antibodies. Our results indicated that the proteins contain at least some carbohydrates. The vector pAcSEcG2T has a thrombin recognition site after the GST sequence that can be used to remove the GST tag by incubating the fusion protein with thrombin. As can be seen in the figure below, incubation of PSG 1 with thrombin for different amounts of time resulted in cleavage of the GST, albeit inefficiently. The 80 KDa protein detected with both, the monoclonal anti-PSG antibody (BAP 1) and the anti GST antibody is the GST-PSG 1 fusion protein released into the supernatant of the insect cells. Cleavage with thrombin resulted in a 53 KDa PSG 1 protein detected only by BAP 1 and the 27-30 KDa GST tag detected only by the anti-GST antibody.



Because the cleavage is so inefficient (notice the amount of fusion protein that remains uncleaved by thrombin even after 22 hours in the figures above) and we need to use very high concentrations of thrombin that can not be easily removed after cleavage, we will use the proteins as GST fusions for our assays. We have recently demonstrated that the fusion proteins bind to macrophages and therefore retain the ability to interact with their receptor. We will scale up the protein purification and test the effect of PSGs in the rate of proliferation of selected breast cancer cell lines. We have also produced a GST fusion protein to be used as control in these experiments. This protein, GST-XylE, is of similar molecular mass and will be produced and purified from the insect cells with the same protocol as the one employed for purification of the recombinant PSGs.

CONCLUSIONS

We have found that after using a very sensitive assay to detect the expression of PSG mRNAs, all cell lines tested which include estrogen receptor negative and positive lines as well as cells derived from normal breast tissue expressed the messages. We used three published primers sets that are specific for PSGs and to increase the sensitivity and specificity of the assay, we Southern blotted the PCR products and hybridized the blots with specific $^{32}P$ labeled oligonucleotide probes. In all cases, we needed to use high number of cycles to detect the PSG mRNAs and therefore we believe that the cells tested do not express high amounts. We will look at more breast cancer cell lines including BT-20, ZR-75-30, and SK-BR-3 for the presence of PSG message in the second year. We have had difficulties obtaining tumor material from NNMC from autopsy specimens for preparation of mRNA, therefore we are testing a method for RNA preparation using formalin-fixed paraffin embedded tissue sections. The sections were obtained from NNMC and include ductal and lobular adenocarcinomas. We will examine the expression of PSGs by PCR as originally proposed and if PSG mRNAs are detected we will proceed in the identification of specific PSGs and splice variants. In a collaboration with Dr. Thompson, we have confirmed the expression of PSGs in 24 out of the 81 breast tumors analyzed and are in the process of examining whether all or specific PSGs are expressed in these tumors. These studies are part of an effort to investigate the role of different members of the carcinoembryonic antigen family in breast tumor prognosis.

We have successfully expressed two PSGs, PSG 1 and PSG 11 in insect cells using a baculovirus expression system. We will scale up the production of the recombinant PSGs and determine their effect in the rate of proliferation of breast cancer cell lines during the second year of this application. As a control we have produced an unrelated GST fusion protein, GST-XylE, that is also produced in insect cells.

PERSONNEL RECEIVING PAY FROM THIS EFFORT:
David Wessner, Ph.D.

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